PB85-218899

Method for Early Life-Stage Toxicity Tests Using Three Atherinid Fishes and Results with Chlorpyrifos

(U.S.) Environmental Research Lab.
Gulf Breeze, FL

1985

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TECHNICAL REPORT DATA
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1. REPORT NO.	2.	3.
EPA/600/D-85/116		PB85-216899

4. TITLE AND SUBTITLE
METHOD FOR EARLY LIFE-STAGE TOXICITY TESTS USING THREE ATHERINID FISHES AND RESULTS WITH CHLORPYRIFOS

5. REPORT DATE
1985

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
L.R. Goodman, D.J. Hansen, D.P. Middaugh, G.M. Cripe, and J.C. Moore

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Same as (12)

10. PROGRAM ELEMENT NO.
ACBE1A

11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS
U.S. Environmental Protection Agency
Environmental Research Laboratory
Office of Research and Development
Gulf Breeze, FL 32561

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA/600/04

15. SUPPLEMENTARY NOTES
Ref: Aquatic Toxicology and Hazard Assessment, ASTM STP 854, R.D. Cardwell et al.,Eds., American Socieity for Testing & Materials, Philadelphia, 1985, pp.145-154

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We have developed methods for obtaining embryos and conducting early life-stage toxicity tests (continuous exposure from the embryonic stage to approximately three weeks or more into the exogenous feeding stage) with three estuarine species of atherinid fishes. Early life-stage tests were conducted for 28-d with Menidia beryllina, M. menidia, and M. peninsulae and the insecticide chlorpyrifos. Responses of the three species were very similar: upper chronic values ("effect" concentrations) ranged from 0.48 to 1.8 µg chlorpyrifos/ℓ and lower chronic values ("no effect" concentrations) ranged from 0.28 to 0.75 µg/ℓ. Chlopyrifos exposure did not affect survival of embryos to hatching which averaged 91 to 93% for each species. In treatments in which no adverse effects were observed, combined survival of M. menidia embryos and hatched fishes averaged 51% and fish weights averaged 23 mg; for M. peninsulae, 69% and 13.6 mg; and for M. beryllina, 81% and 8.7 mg. Bioconcentration factors (concentration in whole body ÷ average measured concentration in water) averaged 220 for M. beryllina, 460 for M. peninsulae, and probably < 420 for M. menidia. From these three species, toxicologists may select an Atlantic or Gulf Coast species that occurs in either high or low salinity.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to public	Unclassified	12
	20. SECURITY CLASS *(This page)* Unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

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Special Technical Testing Publication 854
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1916 Race Street, Philadelphia, PA 19103
1985

EPA/600/D-85/116
1985

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Method for Early Life-Stage Toxicity Tests Using Three Atherinid Fishes and Results with Chlorpyrifos

REFERENCE: Goodman, L. R., Hansen, D. J., Middaugh, D. P., Cripe, G. M., and Moore, J. C., **"Method for Early Life-Stage Toxicity Tests Using Three Atherinid Fishes and Results with Chlorpyrifos,"** *Aquatic Toxicology and Hazard Assessment: Seventh Symposium, ASTM STP 854*, R. D. Cardwell, R. Purdy, and R. C. Bahner, Eds., American Society for Testing and Materials, Philadelphia, 1985, pp. 145–154.

ABSTRACT: We have developed methods for obtaining embryos and conducting early life-stage toxicity tests (continuous exposure from the embryonic stage to approximately three weeks or more into the exogenous feeding stage) with three estuarine species of atherinid fishes. Early life-stage tests were conducted for 28 days with *Menidia beryllina*, *M. menidia*, and *M. peninsulae* and the insecticide chlorpyrifos. Responses of the three species were similar: upper chronic values ("effect" concentrations) ranged from 0.48 to 1.8 μg chlorpyrifos/L and lower chronic values ("no effect" concentrations) ranged from 0.28 to 0.75 μg/L. Chlorpyrifos exposure did not affect survival of embryos to hatching which averaged 91 to 93% for each species. In treatments in which no adverse effects were observed, combined survival of *M. menidia* embryos and hatched fish averaged 51% and fish weights averaged 23 mg; for *M. peninsulae*, 69% and 13.6 mg; and for *M. beryllina*, 81% and 8.7 mg. Chlorpyrifos bioconcentration factors (concentration in whole fish ÷ average measured concentration in water) averaged 220 for *M. beryllina*, 460 for *M. peninsulae*, and probably < 420 for *M. menidia*.

From these three species, toxicologists may select an Atlantic or Gulf Coast species that occurs in either high or low salinity.

KEY WORDS: chlorpyrifos, aquatic toxicology, toxicity tests, early life stage, chronic toxicity, bioconcentration, silversides, *Menidia*, pesticides

The sheepshead minnow (*Cyprinodon variegatus*) early life-stage and life-cycle toxicity tests were developed about a decade ago [*1,2*] and more than 25 of these chronic tests have been conducted [*3*]. Although many investigators

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have determined effects of toxicants on various embryonic, larval, and juvenile stages of marine fishes in acute tests, the only other published marine early life-stage tests that we are aware of are one with longnose killifish (*Fundulus similis*) [*4*] and one with tidewater silversides (*Menidia peninsulae*) [*5*]. Even though application factors for sheepshead minnows and several species of freshwater fishes are usually similar [*3*], comparable early life-stage test methods are needed for more marine species so that we may gain additional insight into relative sensitivities and provide aquatic toxicologists with alternative test species.

In this paper, we report methods for obtaining embryos and conducting 28-day early life-stage toxicity tests with inland silversides (*Menidia beryllina*), Atlantic silversides (*M. menidia*), and tidewater silversides (*M. peninsulae*), and results of exposures to technical-grade chlorpyrifos. In general, methods used were variations of the test method of Goodman et al [*5*] for *M. peninsulae*. *Menidia* spp. are atherinid fishes found in Atlantic and Gulf Coasts estuaries from Newfoundland to Mexico, with some species occurring inland [*6*]. They school and are important forage for larger fishes. Kendall [*7*] stated that their maximum size varies with the species and reported a known maximum length of approximately 13 to 18 cm for *M. menidia*, the largest Atlantic coast *Menidia*. *Menidia beryllina* occurs from Massachusetts to Mexico in saltwater and freshwater [*6*]. *Menidia menidia* is found from Nova Scotia to northeast Florida and has a more saltwater distribution than *M. beryllina* [*8*]. *Menidia peninsulae* occurs from Daytona Beach, Florida, to Horn Island, Mississippi, and from Galveston Bay, Texas, to Tamiahua, northern Veracruz, Mexico [*9*], and is found typically at salinities greater than 20 ppt (parts per thousand). The three species tested are cyclic spawners that produce demersal adherent eggs with diameters ranging from approximately 0.9 mm for *M. beryllina* to 1.2 mm for *M. menidia* [*10–12*].

Chlorpyrifos was selected for our method-development and comparative early life-stage toxicity studies because 96-h LC_{50} values for two cyprinodontids differ markedly (4.1 μg/L for *F. similis* and 136 μg/L for *C. variegatus*) [*13*] and we believed that it would be a good test of the application factor hypothesis [*14,15*]. Also, we had unpublished laboratory data demonstrating that growth of fish can be adversely affected by chlorpyrifos exposure, growth being an important endpoint in the early life-stage method we were developing.

Chlorpyrifos is a chlorinated organophosphate insecticide used to control mosquitoes, fire ants, household insects, turf and ornamental plant insects, and for other purposes [*16*]. Its half-life was 24 (95% confidence interval = 20 to 29) days in a seawater-sediment persistance test [*13*]. In flow-through tests with measured chlorpyrifos concentrations, Schimmel et al [*13*] obtained the following 96-h LC_{50} values: 0.035 μg/L for mysids (*Mysidopsis bahia*), 4.1 μg/L for longnose killifish (*Fundulus similis*), 136 μg/L for sheepshead minnows (*Cyprinodon variegatus*), 1.7 μg/L for Atlantic silversides (*M. menidia*), and 5.4 μg/L for striped mullet (*Mugil cephalus*). Korn and Earnest [*17*] reported

a 96-h LC_{50} of 0.58 μg chlorpyrifos/L for juvenile striped bass (*Morone saxatilis*). In acute tests with five freshwater fish species, 96-h LC_{50} values ranged from 2.4 μg chlorpyrifos/L for bluegill (*Lepomis macrochirus*) to 280 μg chlorpyrifos/L for channel catfish (*Ictalurus punctatus*) [*18*]. Tagatz et al [*19*] found that continuous chlorpyrifos concentrations ≥ 0.1 μg/L significantly reduced the abundance of arthropods colonizing sand-filled aquaria that received unfiltered seawater for eight weeks and that a one-week exposure to 5.9 μg/L significantly reduced the number of arthropods in aquaria colonized in the field for eight weeks and transported to the laboratory prior to dosing.

Materials and Methods

Embryos for the toxicity tests were obtained either by field stripping or laboratory spawning procedures. Adult *M. menidia* were seined from the North Edisto River estuary, South Carolina, and stripped of gametes; eggs were fertilized, entangled on nylon strings by their chorionic fibrils, rinsed, suspended in thermos bottles containing water at approximately 25°C and 25 ppt salinity, and shipped to our laboratory via air express. Alternatively, embryos can be obtained by laboratory spawning procedures that are now available for this species [*10*]. *Menidia peninsulae* and *M. beryllina* embryos were obtained by the laboratory spawning procedures described in detail by Middaugh and Hemmer [*11, 12*]. Brood fish were collected in the vicinity of our laboratory and held in fiberglass tanks (diameter 1.3 m, water depth 38 cm) with recirculating filter-reservoir systems. Brood stocks were maintained at 24 ± 1°C and at salinities of 22 to 27 ppt for *M. peninsulae* and 5 to 7 ppt for *M. beryllina*. Water pumped from the filter reservoir produced mean surface current velocities of 8 cm/s in the tanks. Water flowed from the bottom of each tank through an inverted standpipe and was returned to the filter reservoir system. The gravity-flow filter system contained strata of polyester filter fiber, activated charcoal, coarse pebbles, and crushed oyster shells. Polyester aquarium filter fibers suspended at the water's surface were used as a spawning substrate. Thirty to forty adults (1:1 sex ratio) of the respective species, maintained in each of two brood tanks, provided 600 to 1800 embryos each week. Synchronous spawning occurred when each species was maintained under the influence of an interrupted current velocity cue (18 to 0 cm/s twice daily for 1 h) and a diel light cycle of 13L:11D ("lights-on"—0600 to 1900). *Menidia menidia* spawned at the time of "lights-on" in the morning and also when current velocity was interrupted during daytime [*10*]. *M. peninsulae* spawned shortly after "lights-off" and when current velocity was interrupted during nighttime. *Menidia beryllina* spawned throughout the day and night. *Menidia menidia* embryos and *M. peninsulae* brood stock contained *p,p'*-DDE residues (0.025 and 0.012 μg/g, respectively) but no detectable chlorpyrifos (< 0.020 μg/g) or polychlorinated biphenyls (< 0.10 μg/g). Neither brood stock nor embryos for the *M. beryllina* experiment were chemically analyzed.

Seawater used in toxicity tests with *M. peninsulae* (18 to 25 ppt salinity) and *M. menidia* (18 to 27 ppt) was pumped from Santa Rosa Sound, Florida, through a sand filter and a 20-μm polypropylene filter to a constant-head box where it was aerated and cooled to 25 ± 2°C. Seawater for the *M. beryllina* test was obtained in the same manner except that a 3 to 4 day supply was periodically pumped to a fiberglass storage tank, aerated, and diluted to 4 to 6 ppt salinity prior to delivery to the head box. Freshwater used for dilution was obtained from a municipal well-water supply, filtered with both 20-μm polypropylene and charcoal filters, and stored in a vigorously aerated fiberglass reservoir. A diluter similar to that described by Schimmel et al [*2*] was used in each test. The diluter delivered 1 L of water to each control and chlorpyrifos treatment as each cycle (approximately every 15 min). Triethylene glycol was used as the carrier solvent at a concentration of 7.9 μL/L in the carrier-controls and all chlorpyrifos treatments. Nominal concentrations were seawater-control, carrier-control, 0.12, 0.25, 0.50, 1.0, and 2.0 μg chlorpyrifos/L, except that 0.06 μg chlorpyrifos/L was substituted for the seawater-control in the *M. menidia* test. Splitter boxes equipped with either two or four (depending on which test) 2-mm internal dimension (I.D.) glass capillary tubes slowly delivered the seawater from the diluter to each of the two exposure aquaria for each treatment. A 4-mm I.D. self-starting siphon varied the water depth in each aquarium (21 cm long by 22 cm wide by 10 cm high) between approximately 4 and 7 cm. Exposure aquaria were partially immersed in a freshwater bath that maintained the *M. menidia* test between 25 and 28°C and the other tests at 25 ± 1°C. The photoperiod during testing was 12L:12D.

Silverside embryos have numerous chorionic fibrils and were therefore gently teased apart and their fibrils clipped before selecting those to be used in the toxicity test. Embryos were examined microscopically; groups of four or five (depending on the test) were randomly placed in each incubation cup until the cup contained either 15 (*M. menidia* and *M. peninsulae*) or 16 (*M. beryllina*) embryos. Two incubation cups were then placed in each of the two duplicate aquaria for the seven treatments. Incubation cups were of two types. Wide-mouthed glass jars (8.5 cm in depth and I.D.) with two 2.5-cm holes covered with 363-μm nylon mesh on opposite sides and 1.5 cm from the bottom were used for the first eleven days of the *M. peninsulae* test. Glass petri dish bottoms (9-cm I.D.) with 10-cm-high cylinders of 363-μm nylon mesh attached to the inside walls with silicone adhesive were used for the remainder of this experiment and for the other two species. At test initiation, embryos were approximately 32 to 36 h post-fertilization. *Menidia menidia* embryos were at Stage 18 in development; *M. peninsulae* and *M. beryllina* were at Stages 18 to 20 [*20*].

Observations were made daily for deaths and signs of poisoning. Fry were first observed on Day 4 in tests with *M. menidia* and *M. peninsulae* and on Day 5 in the *M. beryllina* test. Silverside larvae are fragile and susceptible to injury or stranding on the mesh of incubation cups. Therefore, during intervals af-

ter hatching began, the incubation cups were not removed from the exposure aquaria, and larvae were counted in place. Intervals were two days for *M. menidia*, five days for *M. peninsulae*, and ten days for *M. beryllina*. Before incubation cups containing hatched fish were removed from the aquaria, the water level was slowly lowered to below the top of the petri dish using a 4-mm (I.D.) glass siphon.

Rotifers (*Branchionus plicatilis*) and *Artemia* nauplii (24 to 32 h post-hydration of cysts) were used as food for the *Menidia* spp.. *Menidia menidia* were provided a mixture of rotifiers and *Artemia* for the first eight days after hatching began and only *Artemia* until test termination. *Menidia peninsulae* and *M. beryllina* were fed only rotifers for the first seven to eight days after hatching began followed by a three-day transition period in which both rotifers and *Artemia* were provided in three to five feedings per day and then *Artemia* only until test termination. Except during transition periods, fish were usually fed two or more times per day. The number of rotifers provided were estimated to be approximately 2500 per cup per day for *M. menidia*, and were approximately $\geq$ 4500 per cup per feeding for *M. peninsulae* and *M. beryllina*. Approximately $\geq$ 2300 *Artemia* were provided to each cup per feeding. In each test, equal portions of food were dispensed to all cups containing hatched fish except those with few survivors, which sometimes received approximately one half as much when considerable food remained from previous feedings. Fish were not fed on the last day of the test.

At termination of the 28-day experiments, fish were killed in hot water, drained on paper towels, weighed individually, combined within exposure concentrations, and frozen for subsequent chlorpyrifos analysis. Chlorpyrifos concentrations in whole fish and water were determined by the analytical methods of Schimmel et al [*13*], except that approximately 85% of the water samples from the *M. menidia* test were extracted with Amberlite XAD-4 resin.[2] After 1 L of water passed through the resin columns during approximately 1 h, the columns were washed with freshwater and aspirated. Columns were then washed with 5 mL of nanograde acetone to elute chlorpyrifos. After elution, sample preparation and analysis were the same as those of Schimmel et al [*13*]. Recoveries of spiked water samples averaged 97 $\pm$ 14% (N = 15). Water concentrations (Tables 1 to 3) were not adjusted for percentage recovery. Recoveries of spiked tissue samples during analytical method validation averaged 92 $\pm$ 10 (N=13). Dissolved oxygen concentrations were measured approximately weekly, or more frequently, in treatments containing live fish. Treatment means (mg/L) ranged from 7.6 to 7.8 for *M. beryllina*, 5.2 to 5.5 for *M. menidia*, and 6.0 to 6.9 for *M. peninsulae*. Analysis of variance and Duncan's multiple range test were used to analyze survival and weight data. Individual fish weights were used in weight analyses. Percentages were arcsine

[2] Amberlite is a trademark of Rohm and Haas Company, Philadelphia, Pa. Mention of trade names or commercial products does not constitute endorsement by the U.S. Environmental Protection Agency.

TABLE 1—*Survival, average wet weights, residues, and bioconcentration factors (BCF = concentration in whole fish ÷ average measured concentration in water) for inland silversides* (Menidia beryllina) *continuously exposed to chlorpyrifos for 28 days at 25°C.*

Exposure Concentration, μg/L		Survival, %					
Nominal	Measured	Embryos	Fry	Combined	Weight, mg	Residue, μg/g	BCF
Seawater-control	ND[a]	92	86	80	7.9 ± 1.8[b]	ND[a]	...
Carrier-control[c]	ND	94	88	83	8.3 ± 1.8	ND	...
0.12	0.076 ± 0.004[b]	88	87	76	8.6 ± 2.0	ND	...
0.25	0.18 ± 0.008	90	95	86	8.7 ± 2.1	0.019	105
0.50	0.36 ± 0.07	92	95	88	9.1 ± 1.8	0.072	200
1.0	0.75 ± 0.02	92	78	72	9.4[d] ± 1.8	0.10	130
2.0	1.8 ± 0.67	89	47[e]	41[e]	6.6[e] ± 2.1	0.80	440

[a]ND = not detectable (< 0.015 μg/L in water, < 0.010 μg/g in tissue).
[b]Mean ± standard deviation.
[c]Carrier-control and all chlorpyrifos treatments contained 7.9-μL triethylene glycol/L.
[d]Significantly larger than both control treatments ($\alpha = 0.05$).
[e]Significantly less than both control treatments ($\alpha = 0.05$).

TABLE 2—*Survival, average wet weights, and residues in Atlantic silversides* (Menidia menidia) *continuously exposed to chlorpyrifos for 28 days at 25 to 28°C.*

Exposure Concentration, μg/L		Survival, %				
Nominal	Measured	Embryos	Fry	Combined	Weight, mg	Residue, μg/g
Carrier-control[a]	ND[b]	97	43	41	23.4 ± 6.6[c]	ND[b]
0.06	0.079 ± 0.051[c]	83	64	52	22.9 ± 7.1	ND
0.12	0.086 ± 0.040	93	52	48	22.7 ± 8.5	ND
0.25	0.12 ± 0.038	95	64	61	22.8 ± 7.9	ND
0.50	0.28 ± 0.095	96	57	55	23.2 ± 9.8	ND
1.0	0.48 ± 0.14	82	17[d]	15[d]	15.8[e] ± 7.3	ND
2.0	1.1 ± 0.38	88	2[d]	2[d]	12.0[e]	...

[a]Carrier-control and all chlorpyrifos treatments contained 7.9 μL triethylene glycol/L.
[b]ND = not detectable (< 0.015 μg/L in water, < 0.2 μg/g in tissue).
[c]Mean ± standard deviation.
[d]Significantly less than the control response ($\alpha = 0.05$).
[e]Not analyzed statistically due to small sample size and feeding bias.

transformed; a significance level of $\alpha = 0.05$ was chosen for all statistical analyses.

Results

Chlorpyrifos exposure decreased survival of hatched fish but not embryos (Tables 1 to 3). Survival of embryos to hatching was ≥ 80% in all treatments in the three tests. Measured chlorpyrifos concentrations that caused significant reduction in survival of hatched fish were: 1.8 μg/L for *M. beryllina*, ≥0.48

TABLE 3.—*Survival, average wet weights, residues, and bioconcentration factors (BCF = concentration in whole fish ÷ average measured concentration in water) for tidewater silversides* (Menidia peninsulae) *continuously exposed to chlorpyrifos for 28 days at 25°C.*

Exposure Concentration, μg/L		Survival, %				Residue,	
Nominal	Measured	Embryos	Fry	Combined	Weight, mg	μg/g	BCF
Seawater-control	ND[a]	98	90[b]	88[b]	12.1 ± 3.7[c]	ND[a]	...
Carrier-control[d]	ND	95	66	63	13.8 ± 4.0	ND	...
0.12	0.093 ± 0.012[c]	100	80	80	15.1 ± 4.0	0.038	410
0.25	0.19 ± 0.046	93	68	63	13.9 ± 3.0	0.076	400
0.5	0.38 ± 0.11	93	54	51	13.3 ± 4.6	0.22	580
1.0	0.78 ± 0.19	80	31[e]	23[e]	12.3 ± 3.4	...	...
2.0	1.3 ± 0.29	93	5[e]	5[e]	9.0[f] ± 0.8	...	...

[a]ND = not detectable (< 0.015 μg/L in water, < 0.030 μg/g in tissue).
[b]Significantly greater than carrier-control ($\alpha = 0.05$).
[c]Mean ± standard deviation.
[d]Carrier-control and all chlorpyrifos treatments contained 7.9 μL triethylene glycol/L.
[e]Significantly less than both control treatments ($\alpha = 0.05$).
[f]Not analyzed statistically due to small sample size and feeding bias.

μg/L for *M. menidia*, and ≥ 0.78 μg/L for *M. peninsulae*. Most deaths occurred within the first ten days after hatching began. Silversides in chlorpyrifos concentrations that caused significant mortality were observed to be less active than control animals and, during portions of the test, fish in the highest "no effect" concentrations also appeared less active than controls.

Average wet weights of silversides at termination of the 28-day tests were less in the higher chlorpyrifos concentrations than in the controls (Tables 1 to 3). *Menidia beryllina* weights were significantly reduced by continuous exposure to 1.8 μg chlorpyrifos/L. The greater average weights for *M. beryllina* exposed to 0.75 μg chlorpyrifos/L may have resulted from increased food availability because there were fewer survivors (Table 1). Although not analyzed statistically because of the high chlorpyrifos-related mortality and consequent small sample size and feeding bias, average weights of *M. menidia* exposed to chlorpyrifos concentrations ≥ 0.48 μg/L and *M. peninsulae* exposed to 1.3 μg/L were ≤ 68% of their respective carrier-controls.

When chlorpyrifos was detected in silversides, bioconcentration factors (concentration in whole fish ÷ averaged measured concentration in water) averaged 220 for *M. beryllina* and 460 for *M. peninsulae* (Tables 1 and 3). Chlorpyrifos was not detected (< 0.20 μg/g) in *M. menidia* that survived the test, suggesting a bioconcentration factor of < 420 for this species.

Discussion

The lower and upper chronic values (highest concentration that caused no significant adverse effect and lowest concentration that caused a statistically

significant adverse effect) for the three species of *Menidia* are similar. Lower and upper chronic values, respectively, for *M. beryllina* were 0.75 and 1.8 μg chlorpyrifos/L, for *M. menidia*, 0.28 and 0.48 μg/L, and for *M. peninsulae*, 0.38 and 0.78 μg/L. The chronic sensitivity of *M. beryllina*, the least sensitive of the three species tested with chlorpyrifos, is similar to that of the freshwater fathead minnow (*Pimephales promelas*), which has lower and upper chronic values of 1.6 and 3.2 μg technical chlorpyrifos/L [*21*]. Although survival was reduced at all three upper chronic values, average weight was almost as sensitive a criterion of effect. Average weights were a codeterminant of *M. beryllina's* upper chronic value and were markedly reduced, though not analyzed statistically, at *M. menidia's* upper chronic value and at the next exposure concentration above *M. peninsulae's* upper chronic value. In early life-stage toxicity tests with fathead minnows, weight was the most sensitive effect parameter for technical chlorpyrifos and was equally as sensitive as mortality for a chlorpyrifos slow-release formulation [*21*].

Division of the *Menidia* chronic values by their respective 96-h LC_{50} values (obtained in flow-through tests with measured concentrations) of 1.7 μg/L for *M. menidia* [*13*] 4.2 μg/L for *M. beryllina*, and 1.3 μg/L for *M. peninsulae* (James R. Clark,[3] unpublished data) yields estimated application factor ranges of 0.16 to 0.28 for *M. menidia*, 0.18 to 0.43 for *M. beryllina*, and 0.29 to 0.60 for *M. peninsulae*. Application factors for the three *Menidia* species are essentially the same, but are quite different from the estimated application factor range of 0.01 to 0.02 that can be derived from the data of Jarvinen and Tanner [*21*] for fathead minnows exposed to technical chlorpyrifos.

The sequence in which the silversides were tested was *M. menidia*, *M. peninsulae*, and *M. beryllina*. Modifications in experimental design improved survival. On the basis of these results and other unreported tests with these species, we recommend that: (1) incubation cups consisting of a 363-μm nylon mesh cylinder attached inside a 9-cm (I.D.) petri dish bottom be used for all three species; (2) rotifers be used as a first food for all three species; (3) high concentrations of *Artemia* be provided only when the fish can eat them; (4) incubation cups not be removed from the aquaria for approximately five days or more after hatching begins because newly-hatched fish are fragile and susceptible to injury and stranding; (5) young *Menidia* never be netted.

From these three atherinids, toxicologists may select an Atlantic or Gulf Coast species that occurs in either high or low salinities. Techniques for year-round culture, spawning, and testing of each species provide for timely assessment of the effects of riverine, estuarine, and marine pollutants in early life-stage toxicity tests. We can also begin to determine the comparative sensitivities of marine fishes in early life-stage tests.

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Acknowledgments

We thank P. K. Higdon, Dana Christie, and S. D. Friedman for assistance in conducting the toxicity tests, C. H. Deans for statistical analysis of data, Johnnie Knight and Jerrold Forester for chemical analyses, M. J. Hemmer for maintaining the brood fish, and D. M. McMullen for maintaining the rotifer culture. This paper is contribution No. 476 of the U.S. EPA Environmental Research Laboratory, Gulf Breeze, Florida.

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DISCUSSION

Stephen Ells[1] *(written discussion)*—How much effort is involved in setting up and maintaining your rotifer cultures?

L. R. Goodman et al (authors' closure)—Setup and maintenance of a culture system for the rotifer, *Brachionus plicatilis*, can be accomplished with relative ease. The system consists of fourteen 12-L carboys: six are used for growing the green algae, *Chlorella* sp., six for growing rotifers, and two are reserves when cleaning is necessary. Shelf space, gentle aeration, and adequate lighting are required. Maintenance of the system requires 14 to 16 h weekly, with an additional 10 to 12 h every fourth week for cleaning carboys.

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